High Performance nano-LC ESI FT-ICR MS to Verify Disulfide Connectivity and Spin-Labeled X-C GM2 Activator Protein Mutants

Jeremiah D. Tipton[1], Jeff Carter[3], Jordan D. Mathias[3], Mark R. Emmett[1,2], Gail E. Fanucci[3], Alan G. Marshall[1,2]

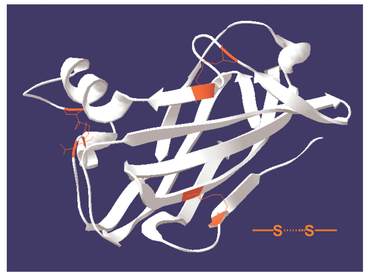
[1]National High Magnetic Field Laboratory, Florida State University, 1800 East Paul Dirac Drive, Tallahassee, FL 32310-4005 USA

[2]Department of Chemistry and Biochemistry, Florida State University, Tallahassee, FL 32306 USA

[3]Department of Chemistry, University of Florida, Gainesville, FL 32611

Introduction

The GM2 activator protein (GM2AP) is a non-enzymatic accessory protein involved in the degradation of neuronal gangliosides. Genetic mutations of GM2AP can disrupt catabolism and lead to deadly lysosomal storage disorders. Crystallography reveals multiple conformations of a loop region in GM2. To investigate crystallography results, we expressed a series of cysteine mutants spin-labeled with MTSL or 4-maleimide tempo (4MT) for EPR analysis. In tandem with other biological techniques, the labeled protein was analyzed with reversed-phase nano-LC, high performance FT-ICR MS, and improved protein informatics to verify spin-labeled X-C GM2AP mutants. Also, these techniques were used to verify 4 disulfide bonds, thus confirming the proper folding of GM2AP for EPR experiments. Presented is the LC/MS assay developed to analyze forthcoming GM2AP mutants.



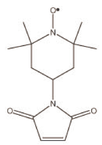
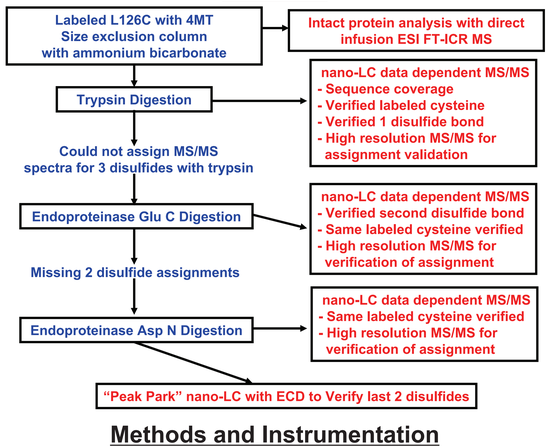


Figure1. Crystal structure of GM2AP with disulfide bonds highlighted in red. Also shown is the structure of 4-maleimide Tempo (4MT).

Workflow



Methods and Instrumentation

Digested samples were analyzed with a modified hybrid linear ion trap FT-ICR MS equipped with a 14.5 T superconducting magnet operated in top-5 data dependent mode. The resulting data was searched with MASCOT (after adding the 4MT mass 253.155, to the modification database). Results from MASCOT were visualized with Scaffold proteomic analysis software. Following the identification of the 4MT labeled peptide, the disulfide bonds were localized to specific peptides by high mass accuracy precursor ion measurements. All relevant peptides were then re-analyzed by nano-LC or direct infusion high resolution MS/MS. Finally, disulfide bonds whose disulfide connectivity could not be localized by CAD MS/MS were subsequently analyzed by "peak parking nano-LC" electron capture dissociation.

Protein Sequence and Calculated Mass

Number of amino acids:
A4 C9 D9 E12 F5 G15 H14 I11 K9 L18 M2 N3 P16 R5 S20 T10 V14 W3 Y4

Molecular formulas and monoisotopic masses:
C893 H1381 N241 O263 S11 (no disulfide bonds): 20040.9023 Da
C893 H1373 N241 O263 S11 (4 disulfide bonds): 20032.8398 Da
C906 H1392 N243 O266 S11 (4 disulfide bonds + Label): 20283.97879

GHHHHHHHHHHSSGHIEGRHMSSFSWDNC8DEGKDP
AVIRSLTLEPDPIVVPGNVTLSVVGSTSVPLSSPLKVDLV
LEKEVAGLWIKIPC68TDYIGSC75TFEHFC81DVLDMLIPT
GEPC94PEPLRTYGLPC105HC107PFKEGTYSLPKSEFVVP
DC126ELPSWLTTGNYRIESVLSSSGKRLGC152IKIAASLKG

Figure 2. Sequence with reported disulfide bonds and amino acid cleavage specificity for a) Trypsin (KR); Glu C (E); Asp N (D). Mutation with label (L126C).

Intact Protein Analysis

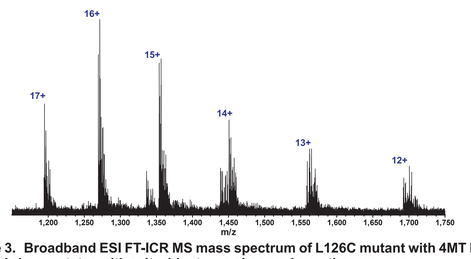


Figure 3. Broadband ESI FT-ICR MS mass spectrum of L126C mutant with 4MT label. Several charge states with salt adducts are observed over the mass range.

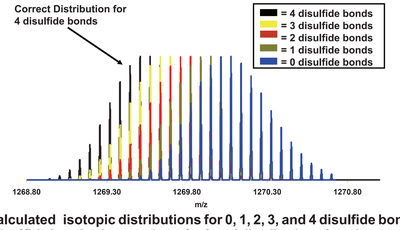
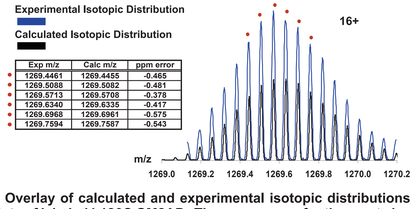


Figure 4. Calculated isotopic distributions for 0, 1, 2, 3, and 4 disulfide bonds. As the number of disulfide bonds change, the calculated distribution also changes.



Exp m/z	Calc m/z	ppm error
1269.4461	1269.4455	-0.465
1269.5088	1269.5082	-0.481
1269.5713	1269.5708	-0.378
1269.6340	1269.6335	-0.417
1269.6968	1269.6961	-0.575
1269.7594	1269.7587	-0.543

Figure 5. Overlay of calculated and experimental isotopic distributions for the 16+ charge state of labeled L126C GM2AP. The mass errors for the most abundant peaks were less than 1 ppm. Based on the fit of the experimental to the calculated isotopic distribution, the protein was predicted to have the correct sequence, with four disulfide bonds, and contained the labeled mutant cysteine.

Digestion with Trypsin

GHHHHHHHHHHSSGHIEGRhmssfswdncdegkDPAVIRSLTLEPDPIVVPG
NVTLSVVGSTSVPLSSPLKVDLVLEKEVAGLWIKipctdyigsctfehfcdvldmli
ptgepcpeplrtyglpchcpfkEGTYSLPKSEFVVPDCELPSWLTTGNYRIESVLS
SSGKrlgcikiaaslkgi

Figure 6. Sequence coverage for the L126C GM2AP mutant after first pass nano-LC MS/MS Top-5 data-dependent analysis. Note that the peptides with disulfide bonds were not observed (in red).

Relevant Tryptic Peptides

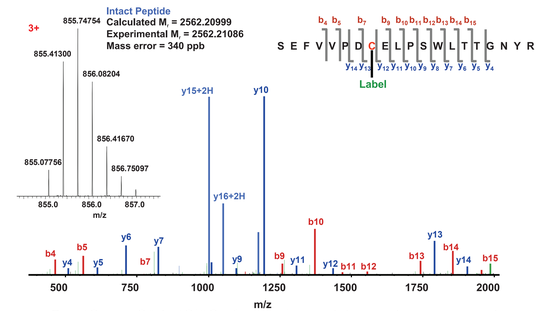


Figure 7. MS and CAD MS/MS for labeled mutated cysteine. The peptide was identified with a simple proteomic workflow with MASCOT and Scaffold.

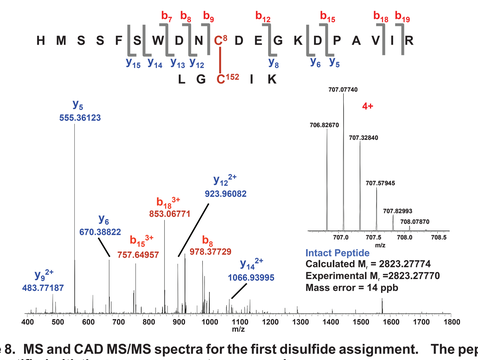


Figure 8. MS and CAD MS/MS spectra for the first disulfide assignment. The peptide was identified with the sequence mass tag approach.

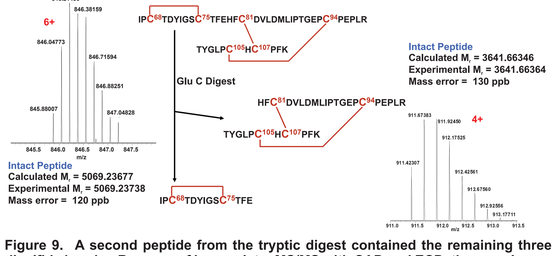


Figure 9. A second peptide from the tryptic digest contained the remaining three disulfide bonds. Because of incomplete MS/MS with CAD and ECD, the sample was digested with GluC.

Relevant Tryptic/Glu C Peptides

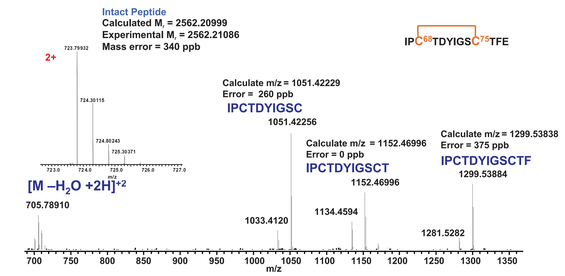


Figure 10. MS and CAD MS/MS for the second disulfide bond. The calculated mass matches the experimental mass for the loss of 2H due to disulfide formation. A sequence tag of three amino acids verified the identity of the disulfide containing peptide. As expected, the cyclic disulfide bond peptide does not yield fragments.

Relevant Tryptic/Glu C/Asp N Peptides

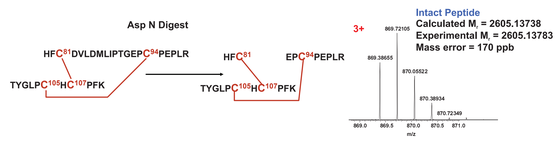


Figure 11. Sequence and disulfide bond connectivity after digestion with Trypsin, GluC, and Asp N. The mass error for the peptide containing the final 2 disulfide bonds was less than 1 ppm.

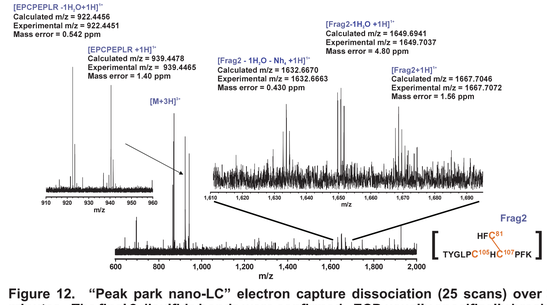


Figure 12. "Peak park nano-LC" electron capture dissociation (25 scans) over 3 minutes. The final 2 disulfide bonds were confirmed. ECD usually specifically breaks disulfide bonds prior to amino acid backbone fragmentation.

Conclusion

A multi-tiered assay has been developed to validate protein folding for future GM2AP mutants, by use of typical proteomic type experiments. High mass accuracy measurements allowed for quick assignment of disulfide connected peptides. Furthermore, high mass accuracy CAD MS/MS aided in the verification of peptide sequence information. In the case in which CAD MS/MS did not establish disulfide connectivity, ECD provided the final evidence. Finally, the present techniques should be easily applied to other in vitro / in vivo posttranslational modification studies.

Acknowledgments

This work was supported by NIH (GM-78359), NSF Division of Materials Research through DMR-0654118, and the State of Florida.